Microbial Pathogenesis 110 (2017) 637–644

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Anti-inflammatory activity of cecropin-A2 from *Musca domestica*

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ARTICLE INFO

Article history:
Received 27 March 2017
Received in revised form
12 July 2017
Accepted 18 July 2017
Available online 19 July 2017

Keywords:
Cecropin
S. aureus
LTA
Proinflammatory cytokines
NFκB
Transcript variant

ABSTRACT

This study aimed to investigate the anti-inflammatory activity of *Musca domestica* cecropin-A2 (Mdc-A2) toward *Staphylococcus aureus* (*S. aureus*) to learn more about their immunological functions. RAW264.7 cells were transfected with recombinant lentiviruses introduce pLEX-Mdc-A2into the RAW264.7 cell line (RAW-Mdc-A2). The RAW264.7 cell line with empty pLEX (RAW-pLEX) was produced in the same manner as a negative control. Real-time quantitative reverse transcription PCR (RT-PCR) was performed to analyze the mRNA expression of TNF-a, IL-1β, NFκB-1 and NFκB-2 in *S. aureus*-stimulated RAW-Mdc-A2 cells and RAW-pLEX cells in untreated cells and cells treated for 3 h, 6 h, 12 h and 24 h. RT-PCR was performed to analyze the mRNA expression of TNF-a, NFκB-1 and NFκB-2 stimulated by Lipoteichoic acid (LTA). Production of TNF-a was detected by enzyme-linked immunosorbent assay (ELISA). Colony counts were used to calculate the number of CFU per mL of cell culture supernatants. The results showed that compared to RAW-pLEX cells, stable transfection of Mdc-A2 in RAW264.7 cells stimulated by *S. aureus* significantly down-regulated the mRNA expression of TNF-a transcript variant 1 (TNF-a-tv-1) at 6 h and 12 h and the mRNA expression of TNF-a transcript variant 2 (TNF-a-tv-2) at 3 h, 6 h and 12 h. Compared to RAW-pLEX cells, stable transfection of Mdc-A2 in RAW264.7 cells stimulated by *S. aureus* significantly down-regulated the mRNA expression of IL-1β-T at 3 h, 6 h and 12 h as well as the mRNA expression of IL-1β at 3 h and 6 h. The expression and production of TNF-a and bacterial burden of cell culture supernatants were significantly down-regulated in RAW-Mdc-A2 cells stimulated by *S. aureus*, and the expression and production of TNF-a were significantly down-regulated in RAW-Mdc-A2 cells stimulated by LTA. Compared to RAW-pLEX cells, stable transfection of Mdc-A2 in RAW264.7 cells stimulated by *S. aureus* significantly down-regulated the mRNA expression of NFκB-1 at 3 h, 6 h and 12 h as well as the mRNA expression of NFκB-2 at 6 h. Additionally, stable transfection of Mdc-A2 in RAW264.7 cells stimulated by LTA significantly down-regulated the mRNA expression of NFκB-1. In conclusion, Mdc-A2 possesses potent anti-inflammatory activity and potent antimicrobial activity. Additionally, Mdc-A2 may interact with LTA and execute strong anti-inflammatory activity by blocking the activation of NF-κB signaling pathways.

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1. Introduction

Antimicrobial peptides (AMPs) are evolutionarily conserved components that are produced as a first line of defense by all multicellular organisms [1]. AMPs are small (3–5 kDa) helical peptides and are produced in bacteria, insects, plants, and vertebrates, and those AMPs can have broad activity to directly kill bacteria, yeasts, fungi, viruses, certain parasites and even cancer cells [2–4]. One of the most notable features of AMPs is that it rarely induces bacterial resistance, which is a serious problem with conventional antibiotics [5,6], and AMPs have low toxicity for mammal cells [7,8]. AMPs have been considered as one of the best potential sources for the development of a new class of antibiotics to be used in the future [9–11]. More than 2810 AMPs have been found in bacteria, archaea, protists, fungi, plants and animals according to the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php).

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http://dx.doi.org/10.1016/j.micpath.2017.07.032
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In recent years, a variety of AMPs have been isolated and classified from different organisms, such as cecropins, histatins, defensins and gloverins [12,13]. Cecropin, which is the first insect AMP to be described, was purified from the hemolymph of the cecropia moth in 1980 [14]. Since then, a large number of insect-derived cecropin AMPs were identified in lepidopteran, dipteran, and coleopteran [15]. Cecropins have a significant potency against both Gram-positive and Gram-negative bacteria [16–18], fungi [19–21], parasites [22,23] and the HIV-1 virus [24]. The mechanism of action of cecropins to kill the bacteria is thought to be pore formation in the lipid membrane of bacteria, which could lead to the disruption of cell membrane integrity [25,26]. House flies, *Musca domestica* L. (Diptera: *Muscidae*), are ubiquitous and transmit more than 100 human and animal diseases [27–29], however, they can thrive without causing infection. The related antimicrobial mechanisms of *Musca domestica* cecropin are thought to damage the bacteria's outer and inner membrane as well as cause leakage of cytoplasmic contents [30]. To date, the cecropin family showed strong anti-inflammatory activity in LPS-stimulated macrophages through interaction with LPS in the cecropia moth *Hyalophora cecropia*, black fly *Simulium bannaense*, horsefly Tabanus yao and swallowtail butterfly *Papilio xuthus* [26,31–33]. However, there are relatively few studies that focus on the anti-inflammatory functions and related signaling pathways in gram-positive bacteria stimulation for *Musca domestica* cecropin. Additionally, the anti-inflammatory activity of the cecropin family was investigated through chemical synthesis and peptide purification. However, few studies have shown whether the expression of *Musca domestica* cecropin in eukaryotic cells has the same anti-inflammatory activity or not.

The trial was conducted to evaluate the anti-inflammatory functions of *Musca domestica* cecropin-A2 (LOC101901181) (Mdc-A2) toward *Staphylococcus aureus* (*S. aureus*) (ATCC 25923). Mdc-A2 was stably transfected into RAW264.7 cells, and the anti-inflammatory activity of Mdc-A2 was established by investigating the mRNA expression of tumor necrosis factor-α (TNF-a), interleukin 1 beta (IL-1β), nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (NFκB-1) and the nuclear factor of the kappa light polypeptide gene enhancer in B cells 2 (NFκB-2) in *S. aureus*-stimulated RAW264.7 cells.

2. Materials and methods

2.1. Cloning and construction of expression vectors for Mdc-A2

Total RNA was extracted from third instar housefly larvae using Trizol reagent (TaKaRa, Japan), and the first strand cDNA was synthesized using PrimeScript™ RT Reagent Kit (TaKaRa, Japan) according to the instructions of the manufacturer. The DNA fragment of Mdc-A2 (XM_005179645.3) peptide was amplified from the cDNA using the primers Mdc-A2-F (ATAA ACG CGT CGC AAA ATG AAT TTC AAT AAA T) and Mdc-A2-R (CTCG ACC GGT TTA AGC GTA GTC TGG GAC GTC GTA TGG GTA ACC CTT TAA TGT GGC GGC AAC ATT AGC TGC CTG CTG GGC CAC ACC AA) in gradient PCR with the following conditions: initial denaturation at 95 °C for 5 min, 16 cycles of denaturing for 30 s at 95 °C, annealing for 40 s at 65 °C (Reduce 2 °C/2 cycles), and extension for 30 s at 72 °C, then 19 cycles of denaturing for 30 s at 95 °C, annealing for 40 s at 51 °C, and extension for 30 s at 72 °C. Primers introduced *Mlu*Iand *Age* I restriction sites, and the underlined parts are the HA tag sequences. Then, both fragments of Mdc-A2 and pLEX (GE, USA) were digested with *Mlu*I(Thermo, USA) and *Age* I (Thermo, USA), and these fragments were gel-purified and cloned. The positive clones were identified by PCR, sequenced, and named pLEX-Mdc-A2.

2.2. The preparation of Mdc-A2 recombinant lentivirus

First, 293 T cells (ATCC, USA) were seeded in a 10-cm culture dish at a density of 2.5×10^6 cells/dish and incubated in DMEM supplemented with 10% FBS (SeraPro, Germany) at 37 °C/5% CO_2 until the cultures grew to approximately 70%–80% confluence. Lipofectamine™ 2000 (Thermo, USA) was used to transfect pLEX-Mdc-A2 into 293 T cells in a 10-cm cell culture dish. Then, 12 μg of pLEX-Mdc-A2 were diluted in 1.5 mL of Opti-MEM® (Gibco, USA) by adding 9 μg packaging plasmid (psPAX2) (GE, USA) and 3.5 μg envelope plasmid (pMD2.G) (GE, USA). Then, the contents were mixed gently. The Lipofectamine™ 2000 was mixed gently before use, then 50 μl were diluted in 1.5 mL of Opti-MEM®.

After the 5 min incubation at room temperature the diluted pLEX-Mdc-A2 was combined with diluted Lipofectamine™ 2000 (total volume = 3 mL). The solution was mixed gently and incubated for 20 min at room temperature. Then, the solution was added drop-wise to 293 T cells cultured in the 10-cm dish. Cells were allowed to incubate at 37 °C/5% CO_2 for 4 h and then the mixture was replaced with 10 mL of fresh DMEM containing 10% FBS. Infectious lentiviruses were harvested at 24 h post-transfection by collecting culture supernatants and filtering them through a 0.45-μm filter. Lentivirus stocks were stored in aliquots at −80 °C until required. The lentivirus containing the empty pLEX vector was produced in the same manner as described above and was used as a negative control.

2.3. Transduction of RAW264.7 cells with recombinant lentiviruses

RAW264.7 cells (ATCC, USA) were cultured in 6-well culture plates at 60–70% confluence on infection day and cultured with DMEM supplemented with 10% FBS. The recombinant lentiviruses and fresh medium (v/v, 1:1) were added with 10 μg/mL polybrene (SIGMA-Aldrich, USA) to the RAW264.7 cells. The cells were further cultured at 37 °C/5% CO_2 for 24 h. After exchanging the solution with fresh DMEM supplemented with 10% FBS, the cells were incubated for an additional 24 h at 37 °C/5% CO_2.

Puromycin (SIGMA-Aldrich, USA) was added to a final concentration of 4 μg/mL in RAW264.7 cells. Fresh puromycin-containing growth medium was exchanged every 2 days. After continuous selection with puromycin. The RAW264.7 cell line containing pLEX-Mdc-A2 was designated RAW-Mdc-A2 and empty pLEX was designated RAW-pLEX as a negative control.

2.4. Western blot analysis

Western blot analysis was performed on cellular protein samples extracted from RAW-Mdc-A2 and RAW-pLEX. The preparation of 40 μg protein samples and Western blot analysis were carried out as previously reported [34]. Approximately 40 μg protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel and transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore, USA). The membrane was blocked by incubating it with 5% BSA (BD) dissolved in Tris-buffered solution Tween-20 (TBST, 2.42 g/L Trisbase, 8 g/L NaCl, 0.1% Tween 20, pH 7.6) for 2 h at room temperature. The primary antibody of the mouse Anti-HA antibody (1:5000, TIANGEN, China) was incubated at room temperature for 1–2 h, and the secondary antibody of the Goat Anti-mouse IgG (1:10000, LI-COR, USA) was incubated at room temperature for 1 h.

2.5. Preparation of RAW-Mdc-A2, RAW-pLEX and S. aureus

RAW-Mdc-A2 and RAW-pLEX cells were cultured in 6-well culture plates (2×10^6/well) and cultured with DMEM

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